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Blue-light Control of Sporangiophore Initiation in *Phycomyces*

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Summary. Many fungi produce spores or spore-bearing structures under the control of blue light. Sporangiophores of *Phycomyces blakesleeanus* are produced continuously along racing tube cultures grown in constant darkness or constant light. However, if a dark-grown culture is exposed to light for a short time on one day a narrow, dense band of sporangiophores is observed the next day at that point of the tube occupied by the mycelial tips during the light pulse. A periodic program with "short days" (*e.g.*, 4 h light; 20 h dark), leads to periodic bands of sporangiophores spaced at intervals corresponding to one period-length (in this case 24 h) of mycelial growth. Sporangiophore initiation is inhibited by a light to dark transition and is stimulated by a dark to light transition. A partial action spectrum of the initiation response, covering the critical 480–540 nm region, strongly suggests that the same photoreceptor pigment is involved as in the phototropic response and light growth response of sporangiophores. Mutants with altered light control of sporangiophore initiation have been found among those selected for altered phototropism. This joint elimination of these two responses to blue light by a single mutation is evidence for a common early transduction system.

Introduction

The fungus *Phycomyces blakesleeanus* produces giant, aerial, spore-bearing structures, called sporangiophores, that are exquisitely sensitive to various stimuli including blue light (Bergman *et al.*, 1969). A change in the intensity of blue-light illumination causes a transient change in the growth rate of the sporangiophore, called the light growth response. An asymmetry in the direction of blue-light illumination causes differential growth in space, called phototropism. Action spectra for both responses have been determined and they are essentially identical (Curry and Gruen, 1959; Delbrück and Shropshire, 1960). Although an exact mechanistic relationship between the responses has not been determined it is clear that differential light growth responses on front and back of the cell underlie phototropism (Castle, 1965; Bergman *et al.*, 1969).

Blue light also controls the growth and development of many other fungi and higher plants (Clayton, 1971). In many well-studied fungal systems blue light synchronizes the production of spore-bearing struc-

tures or the violent discharge of spores (Carlile, 1970; Ingold, 1971). This control of development can be mediated by an internal "clock" (Uebelmesser, 1954; Sargent *et al.*, 1966) or by direct light induction or inhibition at an essential point (Page, 1956; Ingold and Marshall, 1963). A comparison of the action spectra determined for various fungi (*e.g.* Sargent and Briggs, 1967; Gressel and Hartman, 1968; Faraj Salman, 1971) suggests that the photoreceptor pigments involved are similar, and may in fact be identical, to the photoreceptor pigment of the sporangiophores of *Phycomyces*. This observation led me to search for a similar effect of blue light on the sporulation of *Phycomyces*.

This paper describes such an effect, the control by blue light of the initiation of sporangiophores. A partial action spectrum and work with mutants demonstrate that the photoreceptor pigments involved in this response and in the photoresponses of the sporangiophores are identical.

Materials and Methods

Racing Tubes. Rapidly growing cultures of filamentous fungi can be observed for many days by the use of horizontal "racing tubes" that limit the mycelial growth to one dimension (Ryan *et al.*, 1943). Satisfactory growth of the mycelium and sporangiophores of *Phycomyces* was achieved in 25-mm-diameter glass tubing half-filled with solid media. The sterilized medium added to previously sterilized tubes was kept in by dams, produced by indentations of the glass, at each end. The tubes were closed with cotton or foam stoppers. Screw-cap test tubes, 25 × 200 mm, were also used successfully.

Strains. The wild type strain of *Phycomyces blakesleeanus* used in this investigation was NRRL1555, originally obtained from the National Regional Research Laboratories, Peoria, IL., U.S.A. The mutant, C112, was isolated following treatment with nitrosoguanidine and ultraviolet light, by the screening procedure described in Bergman *et al.* (1969). Details on C112 and the other *mad* mutants mentioned are currently available in a thesis (Bergman, 1972) and will be published soon.

Media. The normal racing tube medium used consisted of 40 g/l potato dextrose agar, 1 g/l yeast extract, 0.5 μg/ml thiamin, 7.5 g/l Na_2HPO_4, and 7.5 g/l KH_2PO_4. Agar was sometimes added to make the medium firmer and keep it from moving around in the racing tubes. For some experiments, casein hydrolysate or various individual amino acids were added.

For colonial growth on plates 5 ml/l of 1 N HCl was added to glucose-asparagine agar (Bergman *et al.*, 1969). The colonies were used to inoculate the racing tubes or plates with transfers of a single mycelium.

Light Intensity Measurements. Measurements of blue light and monochromatic light were made with the combination of a 935 phototube (RCA) and a photometer (Eldorado Electronics Model 201 Photometer). This combination was calibrated by comparison with the emission from a calibrated quartz-iodine-tungsten lamp (Cary Instruments, Monrovia, CA, U.S.A.).

For blue light the logarithmic intensity scale originally introduced by Delbrück and Reichardt (1956) is convenient and is used throughout this paper. The logarithms are taken to the base 2 so that each unit corresponds to a doubling of intensity. The unit intensity, $\log_2 I = 0$, is 10 μW cm^{-2} or equivalently 3.8×10^{-11} einstein $cm^{-2}\,s^{-1}$. Monochromatic light intensities are given in einstein $cm^{-2}\,s^{-1}$.

Since the action spectrum for the phototropic response of *Phycomyces* sporangiophores is known the amount of phototropically effective light present in light of various compositions may be determined. Rough estimates of the effective light present in white fluorescent light were made by measuring the intensity with a blue filter (Corning 5–61) over the phototube and then multiplying by 2 to correct for the effective light absorbed by the filter.

Basic Apparatus for Light Programming. A versatile apparatus was needed to subject racing tube cultures to various light programs. In the final design chosen, six compartments (15×27 cm) with separate light-tight covers are contained in one unit which may be closed with a single light-tight cover.

Divergent light beams are reflected and scattered onto the racing tubes from a sheet of white cardboard. Blue light was from a 60-W quartz-iodine-tungsten lamp (General Electric No. 1960) passed through a heat filter (Schott, KG1) and a blue filter (Corning 5–61). Monochromatic light was from a Bausch and Lomb grating monochromator (No. 33-86-25-02) equipped with a 40-W quartz-iodine-tungsten light source (No. 33-86-39-01). The slits on the monochromator were set for a half maximum intensity band width of 10 nm. The illumination was uniform within 10%.

The light programs were controlled by removing and replacing the light-tight covers. When the light-tight covers were removed the compartments were closed with clear plastic sheets. Neutral density filters large enough to cover the compartments were made by flashing sheet film (Kodak Contrast Process Ortho 4154) with diffuse light. White plastic sheets were also used as neutral density filters. The reduction in light intensity produced by these filters was measured *in situ* with a 1P28 photomultiplier coupled to the photometer.

Periodic light-dark programs are indicated by LDx:y, where x hours of light are followed by y hours of dark, x hours of light, *etc.* (Aschoff, 1965).

Growth Conditions. Experiments were routinely started by transferring an agar block containing the whole mycelium from a single spore to a racing tube. After several days in white light from fluorescent lamps (phototropic effectiveness, $\log_2 I = +1$), the tubes were transferred to the experimental apparatus located in a darkroom. The light program was started after at least 12 h in darkness. The position of the mycelial front was marked on the tube at the time of transfer to darkness.

The temperature in the darkroom was $22 \pm 1°$ C (measured with a Serdex recording thermometer, Bachrach Instrument Co., Pittsburgh, PA, U.S.A.). The temperature variation inside the closed compartments was less than 1° C. No attempt was made to measure or control humidity.

Recording of Results. During some of the experiments the position of the mycelial front was periodically marked. This marking was done under red light from lamps equipped with plastic filters (Cinemoid, England) that transmit less than 1% below 560 nm as measured in a Cary 15 spectrophotometer, since preliminary experiments had shown that this light has no effect on the responses studied.

Photographs were taken, at the completion of experiments, on Polaroid Type 55 P/N film using a Polaroid MP-3 camera with diffuse illumination from below.

Plate Assay Technique. For some experiments the racing tubes were replaced with normal, plastic Petri plates containing racing tube medium. Agar blocks with the whole mycelium from a single spore were placed 1 cm from the edge of the plate. After a 96 h light-dark program the sporangiophores in each 0.5×2 cm rectangle of a 16-rectangle grid were counted under a dissecting microscope. The grid was oriented so that the long axis of the rectangles was approximately perpendicular to the direction of mycelial growth. The microscope was used to look

through the tangled mass of sporangiophores and focus on the surface of the agar. The base of each sporangiophore was clearly visible.

Results

Description of Response as Observed

Phycomyces cultures are grown in racing tubes to study periodic growth phenomena over a period of weeks. The tubes are inoculated with a mycelium produced by a single spore. The mycelium grows radially from the point of inoculation until the width of the tube is filled 24–48 h after inoculation. It then grows along the tube with a well-defined front. The front advances at a constant rate of 1.4 cm/day. Since this advance rate is not affected by the light programs used, distance along the tube can be used as a measure of time.

Fig. 1 is a photograph of six tubes taken after they were subjected to periodic light-dark regimes, with the various light and dark periods indicated, for 8 days. A permanent record of periodic sporangiophore initiation is conspicuous in the cultures subjected to periodic regimes with light periods of 8 h or less; dense, narrow bands of sporangiophores alternate with areas of few sporangiophores. The spacing of the bands is determined by the period of the light-dark program. Thus in the third tube the distance between the centers of the bands is 1.4 cm, equivalent to 24 h of mycelial growth.

Nature of Periodicity: Endogenous or Exogenous?

Periodic responses can be produced by the external signal, light in this case, acting directly as a stimulus or indirectly by affecting the phase of an internal clock. The literature documents both types of responses in the fungi. Much of the recent experimental and theoretical work on diurnal rhythms in plants and animals has centered on circadian rhythms, that is, endogenous rhythms with free-running periods close to 24 h (Aschoff, 1965). Consequently, a circadian rhythm is often suspected to be the basis of a newly discovered periodicity. However, the following evidence clearly demonstrates that for the sporangiophore initiation response of *Phycomyces* light acts directly as a stimulus.

1. No periodic production of sporangiophores is observed under constant conditions, either continuous light or continuous darkness (see bottom tube, Fig. 2). Even a culture that has been treated for a week in the LD1:23 program immediately ceases periodic production of sporangiophores on transfer to continuous conditions.

Woodward (1972) has reported that if grown on special media with aeration many wild-type *Neurospora crassa* strains conidiate with a circadian rhythm that was originally found only in special mutant

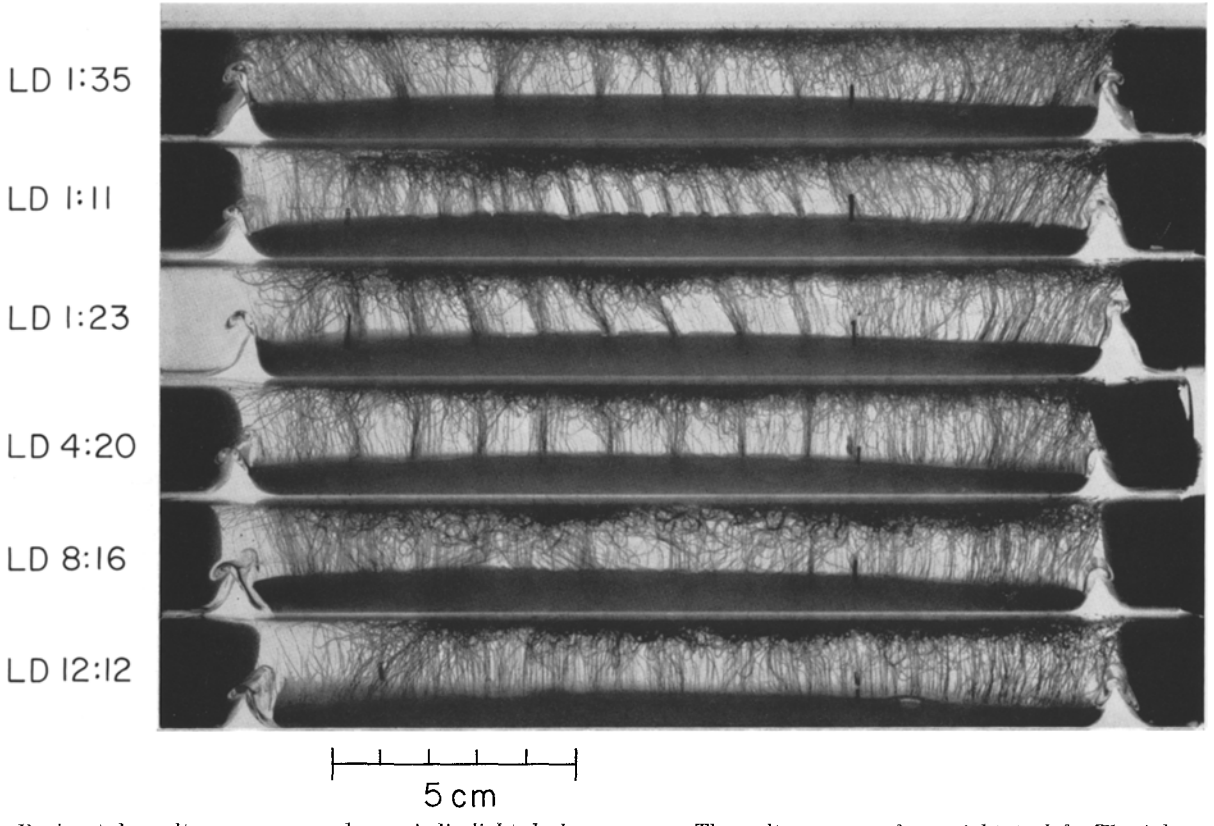

Fig. 1. Racing tube cultures grown under periodic light-dark programs. The cultures grew from right to left. The ink marks at the right of each tube mark the position of the mycelial front at the time transfer to the first dark period. The periodic light-dark programs were started after the first 16-h dark period. The intervals between the 1-h light pulses in the top tube (marked LD1:35) were: 35 h, 11 h, 23 h, 35 h, 35 h. The other five tubes received strictly periodic programs

strains. Prompted by these reports, I tried growing *Phycomyces* cultures on variously supplemented media (casein hydrolysate, various individual amino acids) with and without aeration. In no case was periodicity of sporangiophore initiation visible after transferring cultures to complete darkness.

2. As an additional attempt to demonstrate a "clock" mechanism for the observed periodicity I tested a "double-pulse" program (dark 18 h—light 1 h—dark 4 h—light 1 h). Such a "skeleton" program is equivalent to a LD6:18 program for setting a circadian rhythm in other systems (Pittendrigh, 1965; Minis, 1965). However, the double-pulse program did not cause a single sporangiophore band each day as expected for an LD6:18 program but rather caused two dense sporangiophore bands each day. The bands were each at the position of the mycelial front at the time of a light pulse.

3. The position of a band of sporangiophores (such as those seen in Fig. 1) is at the position of the mycelial front at the time of the light period. This is true even if the length of the dark period is varied from day to day. This direct relationship of the phase of the band with the phase of the signal is clearly different from the type of phase shifting normally observed in the setting of an endogenous rhythm (Pittendrigh, 1965).

Dependence on Light Intensity

Since light is not acting to set an endogenous rhythm it must be acting directly to control sporangiophore initiation. This circumstance facilitates the study of the stimulus-response system since the responses may be a function of the duration of the preceding dark period but not of the phase of an internal "clock".

For a study of the dependence of the response on light intensity a LD1:23 program was used. Blue light was used for the light pulses since preliminary tests showed blue light effective and red light ineffective. Fig. 2 shows the photographic record of the results of a typical experiment. All the cultures were originally grown in white fluorescent light (phototropic effectiveness $\log_2 I = +1$). At the start of the first dark period the position of the mycelial front was marked on each tube. Before the photograph of Fig. 2 was taken black tape was placed with an edge 1 cm back from the mark which was then erased.

The photograph shows that the density of the sporangiophore bands decreases with decreasing light intensity. Apparently, the response saturates at approximately $\log_2 I = -14$ since there is essentially no difference in band density between the top two tubes despite the 40 fold difference in light intensity. At $\log_2 I = -19.2$, bands are no longer clearly discernible by inspection of the photograph. Although an exact

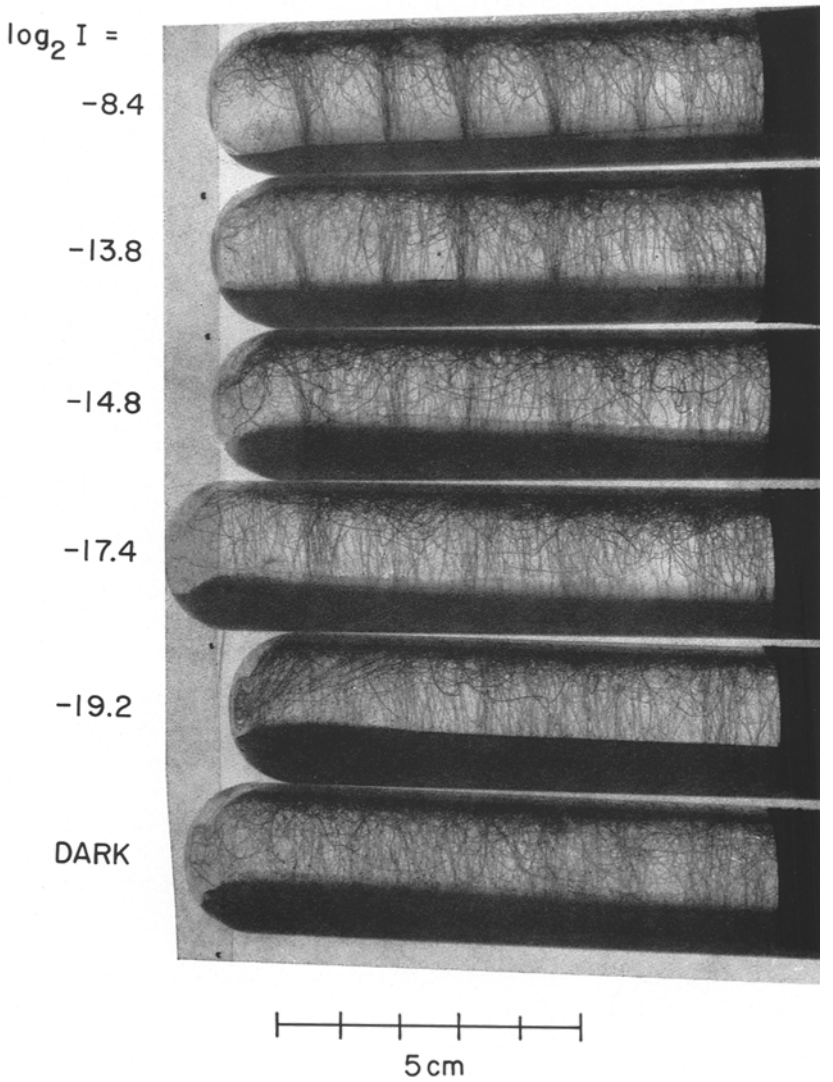

Fig. 2. Light controlled sporangiophore initiation; dependence on light intensity. The cultures in the tubes grew from right to left. Each tube was transferred to the dark when the mycelial front was 1 cm to the left of the tape mark. Following a 13-h dark period 1-h light pulses at the intensities shown to the left of the tubes were given once every 24 h. The spaces between the start of the pulses were: 24 h, 26 h, 22 h, and 24 h. The intensity $\log_2 I = 0$ is equivalent to 3.8×10^{-11} einstein $cm^{-2} s^{-1}$

threshold is difficult to determine it is clear that this response of *Phycomyces* occurs at much lower light intensities than the sporulation responses of other fungi.

In some experiments the total light intensity during a pulse was varied by changing the length of the pulse. Preliminary results, not shown here, suggest qualitatively that it is the total light given during a pulse, of from 1 h down to 15 s, that determines the density of the band.

The results show that for the culture as a whole the response to light (the total density of each sporangiophore band) is graded with light flux and reaches a saturation level at moderate light inputs. At the level of the individual sporangiophore, however, the response must be all-or-none.

Light Induction or Dark Inhibition?

All six tubes of Fig. 2 show a region of inhibition of sporangiophore initiation following the start of the first dark period. In the bottom tube, which was kept in continuous darkness after the transition, continuous sporangiophore initiation began 0.8 cm or 13.7 h later.

Quantitative evidence for this dark inhibition response was obtained by the plate assay technique described in Materials and Methods. In this technique the number of sporangiophores produced per unit area can be counted at the end of the experiment. In Fig. 3 the results of two replicate experiments are shown. There is a clear decrease in the number of sporangiophores following the transition from white light (phototropic effectiveness $\log_2 I = +1$) to darkness. After 1 cm of mycelial growth the inhibition is released and the *number* of sporangiophores produced returns to the original level.

The sporangiophores initiated in the dark seemed to be slightly thinner and less darkly pigmented. This may contribute to the visibility of the response in racing tubes.

Dark inhibition in the number of sporangiophores initiated cannot explain the formation of the dense bands of sporangiophores seen in Figs. 1 and 2. This is shown quantitatively in Fig. 4. The number of sporangiophores initiated in response to the second light pulse shown in this figure is far greater than the average number of sporangiophores initiated in the dark. Thus there must also be a stimulation of sporangiophore initiation or a release from an inhibition in response to a dark to light transition.

Partial Action Spectrum

Since light control of sporangiophore initiation can be caused by low intensities of blue light, it seemed possible that this response might

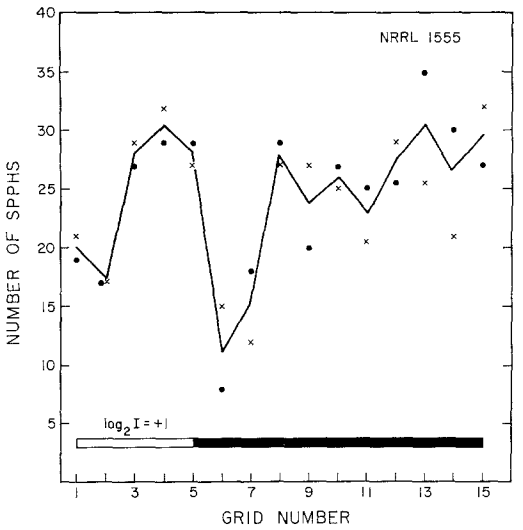

Fig. 3. The effect of a light-to-dark transition on the density of sporangiophore (spph) initiation. The solid line connects the averages of the independent determinations indicated by the two types of points. The light program is shown by the bar at the bottom. The solid black area denotes the dark period

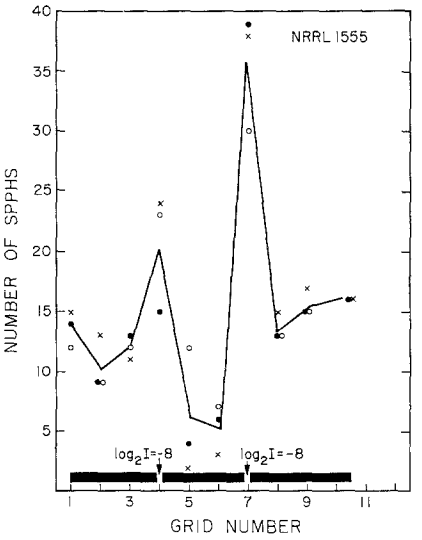

Fig. 4. Light control of sporangiophore (spph) initiation in wild type. The solid line connects the averages of the three independent determinations shown by the points. The light program is shown by the bar at the bottom. The solid black areas denote dark periods. The light pulses, labelled with their intensity, were 1-h long

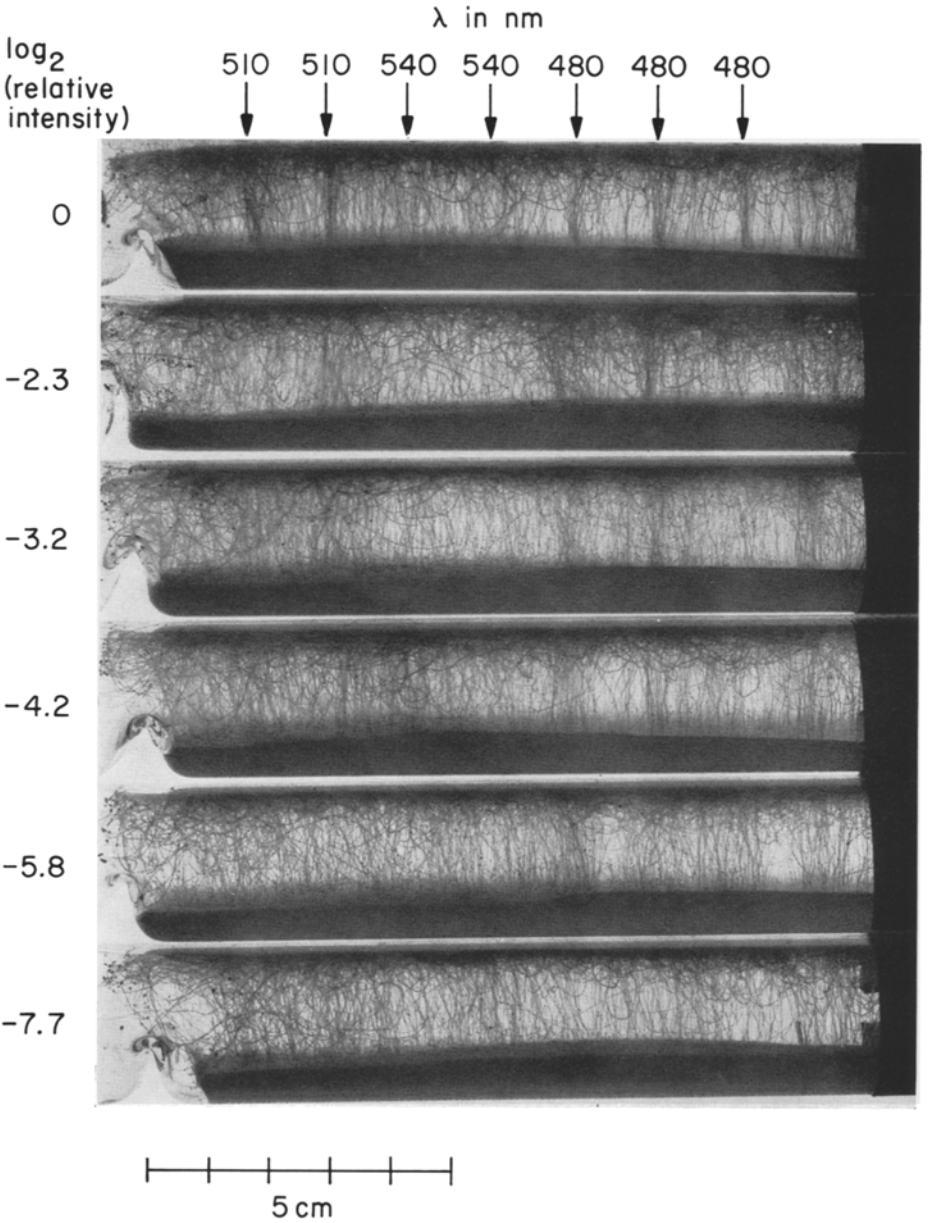

Fig. 5. Comparison of the effectiveness of light of various wavelengths for the control of sporangiophore *initiation*. The cultures, which grew from right to left, were transferred to the dark when the mycelial front was 1 cm to the left of the tape. The 1-h monochromatic light pulses were given exactly 24 h apart.

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share one or more of its sensory transducer elements with the classical phototropic and light-growth responses of sporangiophores.

If the sporangiophore initiation system involves the same primary absorbing pigment as the light response systems of the sporangiophore it should have as identical action spectrum[1]. The accuracy of action-spectrum measurements is limited by the experimentor's ability to quantitate a reference point of the response (for instance, threshold). Since the initiation response can be quantitated only very crudely at this time it would be futile to attempt a detailed action spectrum. Therefore, my measurements were limited to a small spectral region in which the sensitivity of the sporangiophore light responses drops precipitously; the region from 480–540 nm. Fig. 5, a photographic record of the results, shows clearly that for light control of sporangiophore initiation the sensitivity drops off from 480 to 540 nm as well. The response to light of 480 nm in the second tube is approximately equal to the response to light of 510 nm in the top tube. Since the monochromator emits 1.5 times as much light at 510 nm as at 480 nm, light of 480 nm is at least 7.5 times as effective as light of 510 nm. Similarly, 480 nm light is at least 25 times as effective as 540-nm light. These results are close enough to the published action spectra (Delbrück and Shropshire, 1960; Curry and Gruen, 1959) to suggest that the same receptor pigment is active in light control of sporangiophore initiation and the differential growth responses of sporangiophores. It can also be seen that 480 nm and broad-band blue light are approximately equivalent for this response since the threshold in this figure and in Fig. 2 is at 3×10^{-16} einstein $cm^{-2}s^{-1}$.

Mutants

Another proof of the existance of common elements in the transduction pathways involved in the sporangiophore initiation response and the sporangiophore growth responses is provided by the demonstration that both can be eliminated by a single mutation that does not affect growth and morphology. Many mutant strains with altered sporangio-

1 Subject to the usual precautions: (1) the effects of screening pigments are negligible, (2) the quantum yield as a function of wavelength is the same for both responses (Delbrück and Shropshire, 1960).

The final 510-nm pulse was 3 h long. For the top tubes the intensities, denoted by $\log_2$ (relative intensity) = 0, at the wavelengths used were: at 480 nm, 4.5×10^{-15} einstein $cm^{-2}\,s^{-1}$; at 510 nm, 6.8×10^{-15} einstein $cm^{-2}\,s^{-1}$; at 540 nm, 9×10^{-15} einstein $cm^{-1}\,s^{-}$.. The relative light -intensity differences indicated were achieved by the use of neutral density filters. The tubes shown are typical of the five used at each intensity

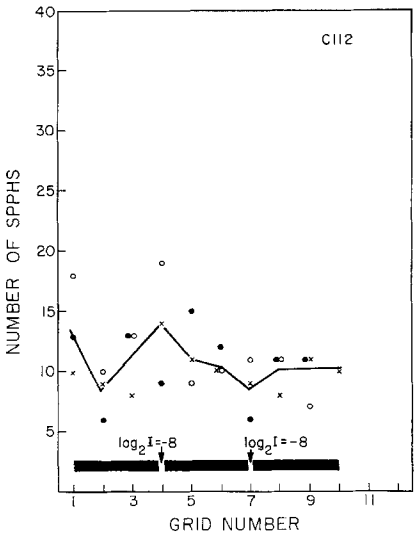

Fig. 6. Light control of sporangiophore (spph) initiation in mutant C112. The solid line connects the averages of the three independent determinations shown by the points. The light program is shown by the bar at the bottom. The solid black areas denote dark periods. The light pulses, labelled with their intensity, were 1 h long

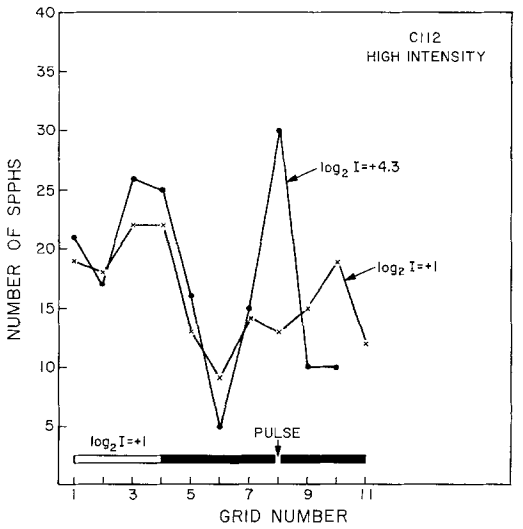

Fig. 7. Light control of sporangiophore (spph) initiation in mutant C112 at high light intensities. Only one determination was made at each pulse intensity. The light program is shown by the bar at the bottom. The solid black areas denote dark periods. The light pulse was 1 h long in each case. The light intensity during the pulses is used to label each curve

phore phototropism (called *mad* mutants) have been isolated and partially characterized (Bergman *et al.*, 1969). Class-1 *mad* mutants require higher light intensities for phototropism (10^7 times higher in one case) but show normal auto-chemotropism, a response (also called the avoidance response) used as a test of the growth output system. For about half of these Class-1 mutants, sporangiophore initiation could not be controlled by light at $\log_2 I = -11$. Fig. 6 shows the results of a plate assay for light control of sporangiophore initiation on the Class-1 mutant C112. This figure should be compared with Fig. 4 which shows the response of wild type to the identical light program. Since at high enough intensities C112 shows normal phototropism, it seemed likely that at high enough intensities light could also control sporangiophore initiation. Fig. 7 shows that C112 shows dark inhibition of sporangiophore initiation on transfer from white light at $\log_2 I = +1$ to darkness and a stimulation of sporangiophore initiation by a 1-h pulse at $\log_2 I = +4$.

Discussion

The development of sporangiophores from the *Phycomyces* mycelium is probably a complex developmental process, involving the production of many new enzymatic and structural molecules. Blue light, it has now been shown, can influence the timing of this event of differentiation in development.

The *response* to light is localized at or near the growing tips since sporangiophores are observed hours later at that point of the culture occupied by the mycelial front during the light pulse. Very likely the *sensitivity* to light resides in the tips, too, but the sensitive region should be more rigorously localized and compared with the site of the response by stimulating with small spots of light. If the site of production of a sporangiophore can be predicted in advance the earliest changes in morphology or protoplasmic streaming could be observed. Preliminary observations have shown that developing sporangiophores are supplied, via a rapid protoplasmic stream, with prefabricated material from a wide area of the mycelium (M. Delbrück, personal communication). This material is probably stored in the "reserve vesicles", dead-end hyphal branches, the first of which develop approximately 25 h after germination of a spore. These first reserve vesicles are emptied at the time of initiation of the first sporangiophores. Interestingly, reserve vesicles seem to accumulate in the dark (Galle, 1964).

Since the initiation of sporangiophores can be triggered to begin at a precise moment, the time of a blue-light pulse can be used as a fixed point in a study of the chemical events that occur at morphogenesis. This fixed point, in conjunction with temperature sensitive mutants of

sporangiophore initiation (Bergman *et al.*, 1969) and the clearly delineated stages of sporangiophore growth, make sporangiophore development a highly attractive model system for the study of differentiation in a eukaryotic microorganism.

It is now clear that two fundamentally different responses of *Phycomyces*, light control of sporangiophore initiation and differential sporangiophore growth in time (light growth response) or space (phototropic response), can be caused by the same stimulus, blue light, and share at least one transducer element. Strains with a single point mutation, that are deficient in both responses, are of special interest since the transducer element altered is a shared and therefore presumably early transducer element. The "sorting-out" power of this method is as yet untested but a collection of mutants blocked in both responses should certainly be enriched for photoreceptor pigment mutants.

Another use of this response is in a search for drugs that affect the light responses of *Phycomyces*. In the past such searches have been hampered by the difficulty of adding drugs to the growing zone of sporangiophores and the intimate connection between the growth rate and photoresponses of sporangiophores. Drugs that affect the photoresponses would be useful if their known mode of action provided clues to the steps involved in sensory transduction. Particularly interesting drugs to try would be those known to interfere with possible intracellular second messengers such as cyclic AMP, acetylcholine, or indoleacetic acid.

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